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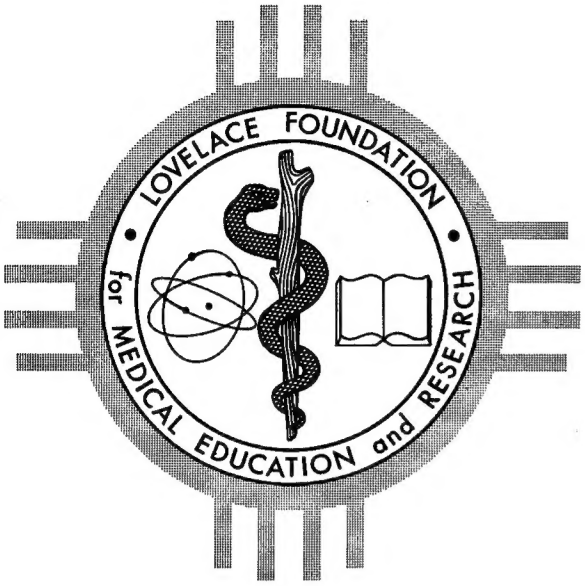

Albuquerque, New Mexico

AEC RESEARCH AND DEVELOPMENT REPORT

UNCLASSIFIED

Physics

FLORA OF HEALTHY DOGS

II. ISOLATION OF ENTEROVIRUSES FROM LOWER INTESTINES

by

W. E. CLAPPER AND F. F. PINDAK

June, 1963

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W. E. Clapper and F. F. Pindak

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ABSTRACT

Fecal specimens from apparently healthy dogs were inoculated into MK, DK, HeLa, and CP cells. Twenty-six cytopathic agents were isolated in MK, 11 in HeLa, and 4 in CP, but none in DK cells. Neutralization tests indicated that all but one of the viruses were either strains of ECHO type 6 or were closely related to it. Twenty-one of 29 dogs were carrying the virus.

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II. ISOLATION OF ENTEROVIRUSES FROM LOWER INTESTINE*

by

W. E. Clapper and F. F. Pindak

INTRODUCTION

There have been few reports of virus isolation from dog feces, and as far as we can determine, none in which agents closely related to human enteroviruses have been encountered. An animal as closely associated with humans as the dog might be expected to harbor some viruses similar to or identical with those found in man. This paper reports multiple isolations from dogs of an agent antigenically similar to ECHO type 6. The investigation was primarily initiated to determine the microbial flora in normal dogs as a part of a program to study the biological effects of inhaling fission products, since such flora could be an important factor in the health of the exposed animal.

MATERIALS AND METHODS

1. Collection of Specimens

All animals were housed in groups of 12 or less in kennels with concrete floors. Their diet consisted of Kasco commercial dry food and water. Specimens were taken from 29 apparently healthy beagles during September through November, 1961. Fifteen were sampled only once. From the remaining 14 dogs, second specimens were collected 29 to 60 days after the first one. Altogether 43 specimens were processed. Plas-

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*This study was presented before the Annual Meeting of the United States - Mexico Border Public Health Association, Nogales, Arizona, April 29, 1963.

tic tubes containing swabs (Falcon), designed to prevent contamination from the outer area of the anus, were used for this purpose. The tubes contained 2 ml of Hanks' BSS with penicillin, streptomycin, and neomycin. Immediately after collection, the fecal material was submerged in this fluid and the tubes were sent to the laboratory without delay for processing. Contents of the swabs were suspended in the above-mentioned solution, and the tubes were inoculated in a 37°C water bath for one hour. Suspensions were then transferred into sterile tubes and centrifuged. Clear supernate was collected and inoculated into tissue culture tubes, or frozen at -20°C, until the culture tubes were available.

2. Tissue Culture Used for Isolation

Approximately 0.1 ml of each supernate was inoculated into (a) primary monkey kidney tissue culture (MK) obtained from Shamrock Farms, (b) primary dog kidney tissue culture (DK) prepared in our laboratory according to the procedure of Younger [1], and (c) HeLa cells. The HeLa cells were originally obtained from the Microbiology Department of the University of Colorado Medical School and had been maintained in our laboratory for more than a year. Sixteen specimens selected at random were also inoculated into a continuous line of cells derived from dog liver (CP), isolated in this laboratory [2]. Growth medium for the HeLa and CP cells was Medium 199 plus 10% calf serum.

The maintenance medium for the MK cells consisted of 50% Earle's BSS, 25% Hanks' BSS, and 25% Medium 199 to which penicillin, streptomycin, neomycin, and mycostatin were added. Maintenance medium for the remaining cell cultures was Medium 199 containing the antibiotics. Media were changed when necessary. Inoculated cultures were incubated at 37°C and observed daily for cytopathic effect for 6 to 8 days. At the end of this period, all cultures were frozen at -20°C and later re-inoculated twice more in the same fashion as the original specimen.

3. Identification Methods

Neutralization tests with known antisera were carried out by observing cytopathic effects on cell sheets in tubes or inhibition of cell metabolism as evidenced by pH changes. Medium used for metabolic inhibition

tests consisted of Medium 199, 5% horse serum, and 1.5 ml of 20% glucose per 100 ml, adjusted to pH 7.6 with $NaHCO_3$. The indicator was phenol red. Penicillin, streptomycin, neomycin, and mycostatin were added to prevent bacterial and fungal growth. MK cells were used in tests where the cytopathic effect was measured. The metabolic inhibition tests were done with CP cells.

RESULTS

1. Number of Isolates Recovered from Each Type of Cell Culture

The results of three successive passages of all 43 specimens in four different cell cultures are summarized in Table 1. Twenty-six cytopathic agents were isolated in MK cells, but none in DK cells. Eleven positive cultures were obtained in HeLa cells. In the CP cells, out of 16 inoculated specimens, four yielded a cytopathic agent. These data may indicate the relative suitability of the four cell lines for isolation of unknown canine enteric viruses. The MK cells, with 60.5% of positive cultures, seem to be by far superior to the other cells in their ability to recover the virus. In contrast, the DK cells, with no isolates, show little promise of successful isolations. The HeLa and CP cells, with 25.6% and 25.0% of positive cultures, appear equally sensitive, but less than the MK cells. The cytopathic changes in MK cells resembled those brought about by growth of human enteric viruses*. All isolates obtained from MK and HeLa cells grew in CP cells to titres sufficiently high to allow the preliminary identification procedures to be carried out in the latter cell line. None of the isolates from MK cells grew in DK cells, although 0.2 ml of undiluted cultures were inoculated.

The relative sensitivity of the four cell lines to recovered viruses is further illustrated in Table 2. Twenty-six viruses were isolated in MK cells: 46.2% of these produced destruction of cells in the second passage and 53.8% in the third. Since the destruction of cells in the primary

*Initial ballooning, followed by granulation and subsequent shrinkage and sloughing from the glass culture tube.

TABLE 1

ISOLATION OF VIRUSES FROM RECTAL SWABS OF DOGS IN VARIOUS CELL TYPES

Dog	Specimen	MK	DK	HeLa	CP
1	a	+	0	0	+
	b	0	0	0	
2	a	+	0	0	+
3	a	+	0	0	
	b	+	0	0	
4	a	+	0	+	
	b	0	0	0	
5	a	+	0	0	+
	b	+	0	0	
6	a	0	0	0	+
	b	0	0	0	
7	a	0	0	0	
	b	0	0	0	0
8	a	+	0	+	0
	b	+	0	0	
9	a	+	0	0	0
	b	0	0	+	
10	a	+	0	0	
	b	+	0	+	0
11	a	+	0	0	0
	b	+	0	0	
12	a	0	0	0	0
	b	0	0	0	
13	a	+	0	+	
	b	0	0	0	
14	a	+	0	0	
	b	+	0	+	

+ = positive virus culture

0 = negative virus culture

MK = primary monkey kidney cells

DK = primary dog kidney cells

CP = continuous line of dog liver cells

TABLE 1 (continued)

ISOLATION OF VIRUSES FROM RECTAL SWABS OF DOGS IN VARIOUS CELL TYPES

Dog	Specimen	MK	DK	HeLa	CP
15	a	0	0	0	
	b	0	0	0	
16	a	+	0	0	
17	a	0	0	0	0
18	a	+	0	0	
19	a	0	0	+	0
20	a	0	0	0	0
21	a	+	0	+	
22	a	0	0	0	0
23	a	+	0	0	
24	a	+	0	+	
25	a	+	0	0	
26	a	0	0	0	
27	a	+	0	+	0
28	a	+	0	+	
29	a	+	0	0	0
Total specimens inoculated		43	43	43	16
Total positive cultures		26	0	11	4
Per cent of specimens giving positive culture		60.5	0	25.6	25

+ = positive virus culture

0 = negative virus culture

MK = primary monkey kidney cells

DK = primary dog kidney cells

CP = continuous line of dog liver cells

TABLE 2

LIST OF POSITIVE SPECIMENS ACCORDING TO CELL TYPES IN WHICH VIRUSES WERE ISOLATED*

Specimen	MK	DK	HeLa	CP
1a	+(2)	0	0	+(3)
2a	+(3)	0	0	+(3)
3a	+(2)	0	0	
3b	+(2)	0	0	
4a	+(3)	0	+(3)	
5a	+(2)	0	0	+(3)
5b	+(3)	0	0	
6a	0	0	0	+(3)
8a	+(2)	0	+(3)	0
8b	+(2)	0	0	
9a	+(3)	0	0	0
9b	0	0	+(2)	
10a	+(3)	0	0	
10b	+(3)	0	+(2)	0
11a	+(3)	0	0	0
11b	+(3)	0	0	
13a	+(2)	0	+(2)	
14a	+(3)	0	0	
14b	+(3)	0	+(3)	
16a	+(2)	0	0	
18a	+(3)	0	0	
19a	0	0	+(3)	
21a	+(2)	0	+(2)	
23a	+(2)	0	0	
24a	+(3)	0	+(2)	
25a	+(3)	0	0	
27a	+(2)	0	+(3)	0
28a	+(3)	0	+(3)	
29a	+(2)	0	0	0
Total	26	0	11	4
Per cent positive in 2nd passage	46.2	0	45.5	0
Per cent positive in 3rd passage	53.8	0	54.5	100

*Numerals in parentheses indicate the passage in which CPE was first observed.

passage, if any, might be attributed to the toxic effect of the fecal material rather than to specific action of a virus, additional passages were made for all specimens. In HeLa cells, 45.5% of the viruses were recovered in the second passage and 54.5% in the third. All four viruses isolated in CP cells destroyed the cells only in the third (and successive) passages. Fourteen specimens gave a positive culture only in MK, two only in HeLa, and one only in CP. Twelve viruses were isolated in both MK and HeLa or CP cells (see Table 3).

2. Recovery of Agents at Second Sampling

Twenty-two of 29 dogs were proven to carry an enterovirus (see Tables 1 and 3). From 15 dogs, specimens were collected only once and virus was isolated from 11. Swabs were taken two different times on the remaining 14 animals. The presence of a viral agent in the stools of these dogs in respect to the time when the specimens were collected is shown in Table 4. Seven dogs (50.0%) had both specimens positive. Four (28.6%) had a positive first specimen, but the second was negative. Three dogs (21.4%) had both specimens negative. None were negative the first time and positive the second time.

3. Identification of Isolates by Neutralization as ECHO 6 Virus

Neutralization of three viruses from MK cells was attempted in CP cells with a pool of antisera for poliomyelitis viruses, types 1, 2, and 3; a pool containing antisera for Coxsackie B, types 1, 2, 3, 4, and 5; and a single Coxsackie B-6 antiserum. All Coxsackie B antisera were obtained from Microbiological Associates (MBA) and the poliomyelitis antisera from the National Foundation for Infantile Paralysis; all were specific for the various types of viruses in the concentrations used. Results shown in Table 5 suggested that the agents belonged to the Coxsackie B group of viruses. One virus (dog number 2) was further tested against individual Coxsackie B antisera. Coxsackie B-4 antiserum was the only one which neutralized 100 tissue culture doses (TCD's) of the virus. However, when neutralization with Coxsackie B-4 antiserum was repeated in MK cells, equivocal results were obtained. Antisera for infectious canine hepatitis and canine distemper viruses failed to neutralize

TABLE 3

DISTRIBUTION OF VIRAL ISOLATES AMONG THE FOUR CELL TYPES

	Total Examined	MK only	HeLa only	CP only	MK and HeLa or CP	DK only
Specimens	41	14	2	1	12	0
Dogs	22	7	1	1	13	0

MK = Primary monkey kidney cells

DK = Primary dog kidney cells

CP = Continuous line of dog liver cells

TABLE 4

RESULTS ON 14 DOGS FROM WHICH TWO SPECIMENS WERE CULTURED

1st and 2nd specimen positive	7
1st specimen positive, 2nd negative	4
1st specimen negative, 2nd positive	0
1st and 2nd specimens negative	3

TABLE 5

PRELIMINARY IDENTIFICATION OF VIRUSES ISOLATED FROM DOGS

METABOLIC INHIBITION IN CP CELLS

	Viruses								
	Coxsackie						Dog		
Antisera	B-1	B-2	B-3	B-4	B-5	B-6	2	18	3a
Anti-polio 1, 2, 3							0	0	0
Anti-Cox. B 1-5 pool (MBA)							+	+	+
Anti-Cox. B-6 (MBA) (1:10)	0	0	0	0	0	+	s1	0	0
Anti-Cox. B-1 (MBA) (1:10)	+	0	0	0	0	0	0		
Anti-Cox. B-2 (MBA) (1:10)	0	+	0	0	0	0	0		
Anti-Cox. B-3 (MBA) (1:10)	0	0	+	0	0	0	s1		
Anti-Cox. B-4 (MBA) (1:10)	0	0	0	+	0	0	+		
Anti-Cox. B-5 (MBA) (1:10)	0	0	0	0	+	0	0		

s1 = slight neutralization

MBA = obtained from Microbiological Associates

dog 2 virus. Intracerebral and intraperitoneal inoculation of this agent into one-day-old mice produced no observable changes. Antiserum was then produced by immunization of a rabbit with tissue cultured dog 2 virus. This antiserum in a 1:40 dilution neutralized dog 2 virus and all but one of the remaining isolates.

Since neutralization in MK cells did not confirm the identification of dog 2 virus in CP cells as Coxsackie B-4, and since neutralizations with ECHO antisera had not been performed, the possibility remained that this was an agent similar to the ECHO viruses. Antisera not being available for all the ECHO types in our laboratory at the time, dog 2 virus was sent to the Viral and Rickettsial Disease Laboratory of the State of California Department of Public Health where it was identified as an ECHO type 6 virus. Confirmation of its identity and the specificity of the rabbit antiserum prepared against it was made in our laboratory by neutralization in MK cells (see Table 6). A 1:1000 dilution of anti-ECHO 6 serum obtained from MBA neutralized 100 TCD's of dog 2 virus. This antiserum in a 1:100 dilution neutralized nine other isolates, indicating that these were also ECHO type 6. Anti-dog 2 serum also neutralized our stock strain of ECHO 6, but not any of the Coxsackie B strains. As shown in Table 6, Coxsackie B strains were all neutralized by specific antiserum obtained from MBA. This proved that the serum produced by using dog 2 virus as antigen was anti-ECHO 6 serum and that it was specific for ECHO 6 virus.

4. One Unidentified Agent Found

One isolate was not neutralized by anti-dog 2 serum. Antisera for Coxsackie B types 1, 2, 3, 4, 5, and 6, ECHO types 1, 2, 3, 4, 5, 6, 7, 8, and 9, infectious canine hepatitis, and canine distemper also failed to neutralize it. In MK cells this virus produced CPE which did not resemble that of other enteroviruses propagated in our laboratory*.

*No ballooning of cells. Stretching out and shredding occurred with no granulation.

TABLE 6

IDENTIFICATION OF DOG VIRUS NUMBER 2. NEUTRALIZATIONS IN MK CELLS.

Antisera	Viruses							
	B-1*	B-2*	B-3*	B-4*	B-5*	B-6*	E-6*	Dog 2
Anti-Cox. B-1 (MBA) 25 units	+**							0
Anti-Cox. B-2 (MBA) 25 units		+						0
Anti-Cox. B-3 (MBA) 25 units			+					0
Anti-Cox. B-4 (MBA) 25 units				+				0
Anti-Cox. B-5 (MBA) 25 units					+			0
Anti-Cox. B-6 (MBA) 25 units						+		0
Anti-ECHO 6 (MBA) 1:100				0			+	+
Anti-ECHO 6 (MBA) 1:1000				0			+	+
Anti-dog Number 2 1:10				0			+	+
Anti-dog Number 2 1:20				0			+	+
Anti-dog Number 2 1:40				0			+	+
Normal rabbit serum 1:10	0	0	0	0	0	0	0	0

+ = neutralization

Coxsackie B and ECHO 6 antisera were obtained from Microbiological Associates.

*Stock cultures, Coxsackie B-1, B-2, B-3, B-5, and B-6 were obtained from Dr. H. A. Wenner of the University of Kansas Medical Center. Coxsackie B-4 was kindly supplied by Dr. Edwin H. Lennette of the State of California Department of Public Health, and the ECHO 6 virus by Dr. E. S. Sulkin of Southwestern Medical College.

**Each Coxsackie B virus and the ECHO 6 virus were neutralized by specific antiserum to prove their identity.

DISCUSSION

Viruses have been isolated from the feces of animals by several investigators. These include an agent cytopathic for bovine kidney from cows and calves [3], enteric viruses in monkeys unrelated serologically to poliomyelitis, ECHO, and Coxsackie groups [4], and enteroviruses from pigs [5,6]. Kunin and Minuse [7] isolated eight agents cytopathic for bovine kidney tissue culture which were unrelated serologically to any of the human enteric viruses. Of several isolates from bovine feces inoculated into MK cells or bovine kidney tissue culture, Moscovici et al [8] found one related to Reovirus type 4. Most of these agents were isolated in bovine kidney, but two were recovered only in MK cells. Klein et al [9] isolated an agent from feces of normal cattle which was related to human adenoviruses. An attempt was made to isolate ECDO viruses from dog feces in dog kidney tissue culture by Gelfand and Flynn [10], but none was recovered although infectious canine hepatitis virus was encountered in specimens from three of 85 dogs.

The relative scarcity of agents in animal feces related to human enteric viruses would appear to be established from these studies. Apparently, no ECHO viruses have been previously isolated from dogs. Yet, one might expect to find these and other human enteric viruses in animals closely associated with man. The agents described in the present paper are antigenically related to the ECHO type 6 virus. Ten of them were neutralized by a known ECHO type 6 antiserum and the remaining isolates, with one exception, were neutralized by anti-dog 2 serum. The neutralization of dog 2 virus initially observed in CP cells with Coxsackie B-4 antiserum will be further investigated.

There seems to be sufficient evidence that the recovered viruses did come from the fecal material of dogs and were not latent simian agents, namely, (1) the "ballooning" of infected cells, with later granulation and complete destruction, is similar to the effect produced by the ECHO and Coxsackie viruses in MK cells and does not resemble the changes brought about by simian viruses, (2) multiple isolations from rectal

swabs of agents which appear to be antigenically identical would seem to indicate that they were present in dog feces, (3) twelve specimens yielding viruses in MK cells gave positive cultures also in HeLa or CP cells, and (4) antiserum prepared against one MK isolate (dog number 2) neutralized not only 24 other viruses isolated in MK, but also 11 isolated in HeLa and 4 in CP cells. Among the latter ones was dog number 2 isolated in CP cells. The antiserum also neutralized a known strain of ECHO type 6.

The virus from dog number 2 was neutralized, in low titres, with sera from dogs closely associated with those from which viruses were recovered. Similar titres of antibodies in dog sera against Coxsackie B-2 were found by Gelfand [11].

It has been reported that dog kidney cells are not susceptible to ECHO viruses types 1, 4, and 9 [12]. We have isolated no agents in dog kidney to date. Those isolated in MK, CP, or HeLa failed to grow in DK cells. It may be possible that the use of cells other than DK was the necessary factor for isolation of these viruses. HeLa cells are generally considered to be unable to support the growth of ECHO viruses, yet the strain used in this work did. A line of HeLa cells (Gey) recently acquired from MBA was not destroyed by an undiluted inoculum of three of the dog viruses which were identified as ECHO 6.

A possible cause for the high frequency of isolation of the same virus may be found in the housing conditions of the animals. They were kept in fairly large kennels, where they certainly came into contact with each other's excreta. Thus, a spread of the same agent from animal to animal was quite possible. We do not attempt to speculate that this virus is a normal inhabitant of the canine intestine.

The dogs showed no symptoms which might be attributed to this virus. However, the frequency of its occurrence indicates the potential capacity of the dog as a carrier of this pathogen and as a possible source of human infection. The presence of such agents in the intestine of

apparently healthy dogs may be of significance in a study of irradiated animals, since exposure to radiation may allow invasion of the virus with resulting physiological changes.

SUMMARY

This study was conducted to determine the viral flora of apparently healthy beagles. The 29 animals examined were housed in groups of 12 or less in kennels with concrete floors. Rectal swabs were taken once only from 15 dogs. From the remaining 14 dogs, a second sample was collected 29 to 60 days after the first one. Altogether, 43 specimens were processed. Necessary precautions were taken to prevent cross- and outside contaminations during the collection of samples. The specimens were treated with antibiotics and inoculated into primary dog kidney tissue culture (DK), primary monkey kidney tissue culture (MK), and HeLa cells. Sixteen specimens, selected at random, were also inoculated into a continuous line of cells derived from dog liver (CP) developed in our laboratory. Three successive passages were done in each cell line.

From 22 dogs, 26 viruses were isolated in MK cells, 11 in HeLa, and 4 in CP cells, but none in DK cells. Thirteen dogs yielded a virus in more than one cell line.

Forty of 41 isolates were neutralized by antiserum prepared against one of them. Other neutralization studies indicated that these viruses were either identical with or closely related to ECHO type 6 virus. The remaining one virus is not related to other isolates. It is neither a canine distemper, nor infectious canine hepatitis virus, nor ECHO types 1, 2, 3, 4, 5, 6, 7, 8, or 9, nor Coxsackie B types 1, 2, 3, 4, 5, 6. Its identity is yet to be determined.

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